ISSN 1330-9862
(FTB-2727)

original scientific paper

The Effect of High Intensity Ultrasound Treatment on the Amount of *Staphylococcus aureus* and *Escherichia coli* in Milk

Zoran Herceg[1], Anet Režek Jambrak[1], Vesna Lelas[1] and Selma Mededovic Thagard[2]*

[1]Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, HR-10000 Zagreb, Croatia

[2]Clarkson University, Department of Chemical and Biomolecular Engineering, 8 Clarkson Avenue, Potsdam, NY 13699-5705, USA

Received: February 2, 2011
Accepted: October 24, 2011

Summary

Inactivation of *Staphylococcus aureus* and *Escherichia coli* in milk containing 4 % milk fat was carried out using a 20 kHz power ultrasound. The experiments were planned and performed according to the statistical experimental design. Specifically, central composite design was used to optimize and design three experimental parameters: temperature (20, 40 and 60 °C), amplitude (60, 90 and 120 μm) and treatment time (6, 9 and 12 min). It was found that Gram-negative bacteria (*Escherichia coli*; $D_{120\ \mu m}$=2.78 min at 60 °C) are more susceptible to the ultrasonic treatment than Gram-positive bacteria (*Staphylococcus aureus*; $D_{120\,\mu m}$=4.80 at 60 °C). Nevertheless, all three parameters studied seem to substantially affect the inactivation of both *Staphylococcus aureus* and *Escherichia coli* in milk using ultrasonic treatment. The results also indicate increased inactivation of microorganisms under longer period of treatments, particularly in combination with higher temperature and/or amplitude.

Key words: milk, high intensity ultrasound, *Staphylococcus aureus*, *Escherichia coli*, response surface methodology

Introduction

Milk is an important source of protein and a worldwide product with high commercial demand. Because it is consumed daily by a large number of consumers, its sensory and microbiological characteristics are of extreme importance. Pasteurization is a common thermal process used to inactivate pathogenic bacteria and some enzymes in milk. However, due to the high temperature used in the process, the nutritional and sensory properties of the pasteurized milk might be somewhat altered. Thus, there is an increased demand for new methods that will have a reduced impact on the nutritional content and the overall food quality.

Recently, several new preservation techniques have been developed that eliminate microbial activity while significantly reducing or completely eliminating the amount of heat required. These processes are, for the most part, less energy-intensive and therefore more cost-efficient and environmentally friendly than conventional thermal processing. Some of the common nonthermal alternatives to conventional thermal processing of foods include pulsed electric field inactivation, pulsed light inactivation, high pressure and ultrasonication (*1*).

Investigation of the ultrasonication as a potential microbial inactivation method began in the 1960s, after it was discovered that sound waves used in anti-submarine warfare killed fish (*2*).

When sound waves pass through a liquid, in each part of the liquid consecutive compression and rarefaction (expansion) cycles take place. Because the acoustic energy cannot be absorbed by molecules during the

*Corresponding author; Phone: ++385 1 460 5037; Fax: ++385 1 460 5072; E-mail: zherceg@pbf.hr

rarefaction cycle of the sound wave, the liquid is pulled apart, which generates a void and induces cavitation, the formation of small gaseous (micro) bubbles. The cavitation process is characterized by two stages: (*i*) rapid increase in the size of microbubble, and (*ii*) fast bubble collapse and the formation of a 'hot spot', high-temperature residual bubble. The implosive collapse of the bubble raises the local temperature to at least 5500 °C and pressures up to 50 kPa. Consequently, under these extreme conditions, vapourized molecules are dissociated into free radicals and rapidly cooled (10^9 °C/s). The collisions between the collapsing bubbles result in the formation of shock waves. Clearly, the effect the ultrasound will have on the treated solution will depend on the number and intensity of bubble implosion per unit of time, the characteristics of the treatment and the characteristics of the treatment media (*3*).

When microorganisms are present in the bulk liquid, microbial killing is believed to occur due to the thinning of cell membranes, localized heating and the production of free radicals (*4–12*).

As an example, microbial inactivation using ultrasound has been investigated for application to a range of liquid foodstuffs. Levels of *E. coli* O157:H7 were reduced by 5 log CFU/mL with ultrasound and mild heating in apple cider (*13*) and the inactivation of *E. coli* K12 was enhanced using ultrasound at ambient temperature (*14*). Dehghani (*15*) investigated the effectiveness of sonication as a disinfection method and found a strong influence of ultrasound on the disruption of *E. coli* in water. In milk and under mild heating, D'Amico *et al*. (*13*) found that ultrasound reduces the levels of *Listeria monocytogenes* by 5 log CFU/mL. Knorr *et al*. (*16*) evaluated the effects of continuous flow ultrasound combined with temperature treatment on bacterial decontamination (*E. coli* and *Lactobacillus acidophilus*) of model suspensions and various liquid food systems including milk, fruit and vegetable juices. The results were compared with conventional heating where it was shown that ultrasound-assisted thermal processing of liquid food can be achieved at lower temperatures and result in further quality advantages.

The objective of this work is to investigate the effect of high intensity ultrasound on the amount of *Staphylococcus aureus* and *Escherichia coli* in milk after ultrasonic treatment. The effect of various parameters (treatment time, amplitude and temperature) on the inactivation of the bacteria was studied experimentally and according to the statistical experimental design.

Materials and Methods

Milk samples

Prior to use, raw cow's milk with 4 % of milk fat was kept refrigerated at 4 °C. The initial microbial load of *S. aureus* and *E. coli* was tested in all milk samples: raw milk (R), sonicated (20 °C) or thermosonicated (40 and 60 °C) (A1–A16). All analyses were performed immediately after ultrasound treatment, and they were all done at least in triplicate for each experiment.

Microbiological analysis

The load of *E. coli* and *S. aureus* in raw and sonicated milk was determined in undiluted samples or in serial dilutions in peptone water (0.1 %). *E. coli* were enumerated by spreading the decimal dilution of 0.1 mL of the sample using the Dragalsky stick on the surface of chromogen medium in Petri dish (Coli ID, bioMérieux, Marcy l'Etoile, France). The samples were incubated at (44±1) °C for 24 h. The result (in CFU/mL) was calculated by counting the red colonies (*17*).

S. aureus were enumerated by spreading the decimal dilution of 0.1 mL of the sample using the Dragalsky stick on the surface of Baird Parker agar. The samples were incubated at (37±1) °C for 24 h. The result (in CFU/mL) was calculated by counting the colonies (*18*). Microbiological analysis was performed after ultrasonication or thermosonication. All microbiological analyses for each batch were conducted at least in triplicate for each experiment.

Ultrasound treatments

Raw milk (200 mL) was placed in a double-walled vessel (200 mL), which served as the treatment chamber. An ultrasonic processor (S-4000, Misonix Sonicators, Newtown, CT, USA), set at 600 W, 20 kHz, and 12–260 μm with a 12-mm diameter probe, was introduced into the vessel. The same part of the probe was immersed in the milk (about 2 cm) and placed at the 'centre' of the sample. Ultrasonications were carried out with 60, 90 and 120 μm amplitude at the temperature of 20 °C. The raw milk samples were treated by ultrasound for 6, 9 and 12 min. For the thermosonication experiments, all the samples were heated at the temperature of 40 or 60 °C before the ultrasonic treatment. Overheating of the samples during sonication was prevented by water cooling of the treatment chamber. The final temperature of milk after sonication at 40 or 60 °C was ±1 °C.

Determination of acoustic power and efficacy of ultrasonic treatments in terms of the elimination of microbes

The most widely accepted method for determining the power of an acoustic horn in an aqueous solution is the calorimetric technique described by Margulis and Margulis (*19*). This method involves taking a known volume of water and applying ultrasound (for ~3 min) while monitoring the change in temperature with time at various ultrasonic amplitudes. The ultrasonic power P and the ultrasonic intensity AI can be readily determined from the following equations:

$$P = m \cdot C_p \cdot \frac{\Delta T}{\Delta t} \qquad /1/$$

$$AI = P/A \qquad /2/$$

where P is the ultrasonic power (W), m is the mass of the sample (kg), C_p is the specific heat capacity of milk (kJ/(kg·°C)), AI is the ultrasonic intensity (W/cm^2), and A is the surface area of the probe (cm^2).

The efficiency of ultrasonic treatment in terms of microbial reduction was measured by the decimal reduction time (D). D value is defined as the time (min)

required to reduce the number of viable cells by one log cycle or the time required to kill 90 % of population at a given temperature and sonic wave amplitude. *D* values were calculated from the slope of the regression line obtained from the straight portion of the survival curve of the counts (CFU/mL). In this study, the *D* value at 20 kHz was abbreviated as D_{us}.

$$\log\frac{N_1}{N_0} = -\frac{t}{D_{us}} \qquad /3/$$

where N_0 is the number of *S. aureus* or *E. coli* before the ultrasonic treatment, N_1 is the number of *S. aureus* or *E. coli* after the ultrasonic treatment at time *t* and D_{us} is the decimal reduction time (min).

Experimental methodology

In order to determine the influence of the operational parameters on the count of *S. aureus* and *E. coli*, central composite design (CCD) (STATGRAPHICS Centurion**,** StatPoint Technologies, Inc, Richmond, VA, USA) and face-centred model were used (*20*). Because CCD requires the choice of operational parameters, the authors have chosen to study the effects of amplitude (μm), temperature (°C), and treatment time (min). Analysis of variance (ANOVA) was carried out to determine any significant differences ($p<0.05$) among the applied treatments. The operating variables were considered at three levels, namely low (–1), central (0) and high (1). Accordingly, 16 experiments were conducted organized in a factorial design (including factorial points, axial points and centre point), and the remaining experiment involving the replication of the central point to get a good estimate of experimental error. Response (output) values were total *S. aureus* and *E. coli* count in log CFU/mL.

The designs were based on a two-level full factorial design and augmented with centre and star points (*21–23*). The total number of experiments of the designs (*N*) was calculated as follows:

$$N=N_i+N_o+N_j \qquad /4/$$

where $N_i=2^n$ is the number of experiments of the two-level full factorial design, N_o is the number of centre points, and $N_j=2\cdot n$ is the number of star points.

Response surface methodology

The experimental results were analyzed by response surface methodology (RSM) using the STATGRAPHICS Centurion software. Specifically, the RSM was used to study the effect of three different ultrasonic parameters: amplitude – X_1 (μm), temperature – X_2 (°C) and treatment time – X_3 (min). In order to optimize the ultrasonic treatment and to investigate the effects of the three independent variables on the count of *S. aureus* and *E. coli*, a central composite rotatable design with the variables at three levels was used in the experiments (Table 1). Design matrix for the experiment as well as the regression model proposed for the response is given by the following equation (*24*):

$$Y=\beta_0+\sum_{i=1}^{4}\beta_i X_i+\sum_{i=1}^{4}\beta_{ii} X_i^2+\sum_{i<j}^{4}\beta_{ij} X_i X_j \qquad /5/$$

where β_0 is the value of the fixed response at the central point of the experiment (point 0, 0, 0); β_i, β_{ii} and β_{ij} are the linear, quadratic and cross-product coefficients, respectively (*25*). The model was fitted by multiple linear regressions (MLR). The validity of the quadratic empirical model was tested with analysis of variance (ANOVA). The confidence level used was 95 %.

Table 1. Treatment time, amplitude, temperature, intensity and added energy during ultrasound treatments

Samples	Amplitude	Treatment time	Temperature	Acoustic intensity	Energy
	μm	min	°C	W/cm^2	J
R	–	–	–		
A1	60	12	20	53.98	58.548
A2	90	6	40	29.84	15.527
A3	120	6	20	27.84	21.521
A4	120	6	60	24.97	19.145
A5	90	9	60	50.08	38.136
A6	60	6	60	26.06	13.436
A7	120	12	60	54.59	60.125
A8	90	9	40	49.85	36.836
A9	60	9	40	41.13	28.392
A10	90	12	40	55.13	62.873
A11	120	12	20	57.49	64.348
A12	90	9	40	49.85	36.836
A13	60	6	20	24.99	12.763
A14	90	9	20	49.16	37.011
A15	120	9	40	52.16	43.084
A16	60	12	60	52.26	64.423

Results and Discussion

The amount of *Staphylococcus aureus* and *Escherichia coli* in milk after ultrasonic treatment was analyzed by response surface methodology (RSM) using the STATGRAPHICS Centurion software**.** According to the RSM model, the count of *S. aureus* in milk (SA) can be described by a polynomial whose parameters are denoted as TT (treatment time, min), T (temperature, °C) and A (amplitude, μm):

$$SA=4.05684-0.011867\cdot A-0.213003\cdot TT-0.0174336\cdot T+ \\ +0.000048659\cdot A^2+0.0000694444\cdot A\cdot TT-0.00000625\cdot A\cdot T+ \quad /6/ \\ +0.00542146\cdot TT^2-0.000645833\cdot TT\cdot T+0.000121983\cdot T^2$$

The predicted model for the count of *E. coli* in milk can be described by the polynomial:

$$EC=5.51044-0.0150606\cdot A-0.411606\cdot TT-0.034344\cdot T+ \\ +0.0000559387\cdot A^2-0.0000138889\cdot A\cdot TT+0.0000479167\cdot \quad /7/ \\ \cdot A\cdot T+0.0139272\cdot TT^2+0.000395833\cdot TT\cdot T+0.000100862\cdot T^2$$

where EC is the count of *E. coli*, TT is the treatment time (min), T is temperature (°C) and A is the amplitude (μm).

The initial count of *S. aureus* in raw milk before the processing was 2.10 log CFU/mL. According to the national sanitary standards, the acceptable amount of *S. aureus* in the pasteurized milk is less than 1.0 log CFU/mL (*26*).

According to Table 2, after ultrasonic treatment of milk containing *S. aureus* at 20 °C, the plate count of sample A3 was reduced for about 0.22 log CFU/mL, whereas the count of sample A13 was 0.09 log CFU/mL. Significant difference in the plate count reduction of *S. aureus* between the two samples is a result of different amplitudes of the applied ultrasound. Overall, this result demonstrates that ultrasonic treatment at ambient temperature (20 °C) for 6 min is not effective enough to reduce the count of *S. aureus* to the level determined by the Regulation for quality of milk and milk products (*26*). However, even though an increase in the treatment time from 6 to 9 (A14) and 12 min (A1 and A11) increased the reduction of *S. aureus*, the final count in those samples was still higher than the one required by the regulations.

Table 2. Counts of *Staphylococus aureus* and *Escherichia coli* after ultrasound treatments

Samples	*Staphylococus aureus*		*Escherichia coli*	
	Count	Reduction	Count	Reduction
	log CFU/mL	log CFU/mL	log CFU/mL	log CFU/mL
R	2.10	–	3.60	–
A1	1.32	0.78	1.37	2.23
A2	1.71	0.39	1.68	1.92
A3	1.88	0.22	2.26	1.34
A4	1.43	0.67	1.33	2.27
A5	0.83	1.27	0.73	2.87
A6	1.63	0.47	1.47	2.13
A7	0.61	1.49	0.53	3.07
A8	1.21	0.89	1.16	2.44
A9	1.35	0.75	1.29	2.31
A10	0.79	1.31	0.78	2.78
A11	1.16	0.94	1.11	2.49
A12	1.21	0.89	1.16	2.44
A13	2.01	0.09	2.06	1.54
A14	1.67	0.43	1.56	2.04
A15	1.14	1.00	1.02	2.58
A16	0.73	1.37	0.62	2.98

Next set of experiments demonstrated that only higher temperatures can lead to an increase in the reduction of *S. aureus* in milk. Thus in samples A5, A7, A10 and A16 (Table 2) the plate count of *S. aureus* was 0.83, 0.61, 0.79 and 0.73 log CFU/mL respectively, which is below the maximal acceptable limit determined by the Regulation.

Maximal inactivation of *S. aureus* in milk occurred after a 12-minute ultrasonic treatment at 120 μm amplitude and 60 °C (sample A7) (Table 2). Some authors have suggested that the efficiency of the ultrasonic treatment for killing the bacteria by cavitational effects could be reduced with an increase in temperature (*10–12*). This trend could be the result of an increased thermal effect that either hinders the effect of sonication or decreases the violence of the bubble implosion due to the increased vapour pressure at higher temperatures (*9,27*). However, this behaviour is not in agreement with the results shown in Table 2. Although the cavitation effect could be minimised by an increase in temperature, in the case of milk, the presence of solids in the suspension could also play an important role and improve the cavitation intensity (*7,9,28*). Garcia *et al.* (*29*), for example, observed that high temperatures improved the effect of ultrasound treatment in killing the bacteria in milk since the z-values of thermosonication and thermal destruction were very similar. Although the mechanism was not clear, the results were attributed to the concentration of solids present in milk. In another study, Ciccolini *et al.* (*30*) investigated the survival of *S. cerevisiae* suspended in water at 45, 50 and 55 °C at different ultrasonic powers. They found that the application of ultrasonic waves at the non-lethal temperature (45 °C) did not display the deactivation while the higher temperatures indicated the synergy between the ultrasound and the heat.

The initial count of *E. coli* in raw milk before the ultrasonic processing was 3.60 log CFU/mL. After treating the milk for 6 min at 20 °C, the initial count of *E. coli* was reduced to 2.26 (A3) and 2.06 log CFU/mL (A13) (Table 2). As it was the case with *S. aureus*, the level of *E. coli* inactivation was enhanced at higher temperatures (40 and 60 °C) and higher amplitudes.

Specifically, the initial counts of *E. coli* were reduced between log 3.98 and log 4.27 after a 12-minute treatment at 60 °C, depending on the wave amplitude (Table 2). This result is in agreement with the findings of Cameron *et al.* (*5*) in terms of the reduction of *E. coli* (log 4.42) achieved after ultrasonic treatment of milk. Contrarily, D'Amico *et al.* (*13*) reported a log 4.63 reduction in viable *E. coli* cells after 6 min of sonication at 20 °C, which was not observed in this study. However, they used a higher frequency (24 kHz), larger probe (22 mm in diameter) and a smaller sample volume in their study. These differences may account for the different reduction values reported in this study.

According to the equation given by Margulis and Margulis (*19*), the value of ultrasonic intensity *AI* (defined as the power of probe per unit of the probe area, W/cm^2) can be used to determine the effect of the cavitation and microstreaming on the inactivation of *E. coli* and *S. aureus* in milk. Thus, by the comparison of the acoustic intensity (*AI*) values in Table 1 with the results given in Table 2, direct correlation between the extent of deactivation and the ultrasonic intensity can be seen. Similarly, the comparison of the *AI* values with the *D* values given in Table 3 demonstrates that the time for decimal reduction (D_{us}) at the specific amplitude (60, 90 and 120 μm) of ultrasound is proportional to the applied intensity. At the lowest temperature (20 °C), the *D* values were between 14.27 and 13.73 for *S. aureus* and 11.21 and 10.80 for *E. coli*, depending on the applied wave amplitude (60, 90 or 120 μm). When the treatment temperature was increased to 40 °C, compared to 20 °C, *D* values for the thermosonication decreased by approx. 25 % for *S. aureus* and 20 % for *E. coli*. The lowest decimal reduction time in the ultrasound treatment at the amplitude of 120 μm (D_{120}) and temperature of 60 °C was 4.80 min for *S. aureus* and 2.78 min for *E. coli* (Table 3), when the intensity of the applied ultrasound was maximal at 57.49 W/cm^2 but delivered energy was 64.423 J (Table 1). Very little information is found in the literature on the influence of the wave amplitude on microorganism inactivation. Nevertheless, it has been reported that the intensity of ultrasound is directly related to the amplitude: when ultrasound amplitude increases, the zone undergoing cavitation increases, leading to more inactivation (*29,31,32*).

Table 3. *D* values after ultrasound treatments at amplitudes of 60, 90 and 120 μm for *Staphylococus aureus* and *Escherichia coli*

Microbe	Temperature °C	Decimal reduction time min		
		D_{60}	D_{90}	D_{120}
Staphylococus aureus	20	14.27	14.07	13.73
	40	10.67	10.59	10.38
	60	4.83	4.85	4.80
Escherichia coli	20	11.21	11.30	10.80
	40	9.04	8.93	8.88
	60	2.95	2.80	2.78

Scherba *et al.* (*33*) did not find any differences in the resistance to ultrasound between the Gram-negative, rod-shaped bacteria and the Gram-positive, coccus-shaped bacteria. In contrast, some authors have suggested that Gram-negative bacteria are more sensitive than Gram-positive (*7,10*). The results of this study demonstrate that Gram-negative bacteria (*E. coli*; $D_{120\ \mu m}$=2.78 min at 60 °C) are more susceptible to the ultrasonic treatment than Gram-positive (*S. aureus*; $D_{120\ \mu m}$=4.80 at 60 °C) (Table 3). Gram-positive bacteria (*S. aureus*) usually have a thicker and more tightly adherent layer of peptidoglycan than Gram-negative (*E. coli*) bacteria, and this morphological feature did seem to be a differentiating factor in ranking the microorganisms according to the percentage of bacteria killed by ultrasonic treatment (*6*).

The estimated effects of each operating variable and an analysis of variance for the model are presented in Tables 4 and 5. According to the ANOVA results, the fitted model was significant at considered confidence level since the F-value was more than three times higher than that of the listed F-value. In order to determine the significance of the effect, the values of the p-value in Tables 4 and 5 need to be observed. Indeed, a p-value lower than 0.05 indicates that the considered factor is significant for the count of *S. aureus* and *E. coli* in milk. Surface plots showing the counts of *S. aureus* and *E. coli* as a function of the treatment time and the amplitude are given in Figs. 1 and 2, respectively. It can be ob-

Table 4. Analysis of variance (ANOVA) for ultrasound treatments and viability of *Staphylococcus aureus*

Source	Sum of squares	df	Mean square	F-value	p-value
A: amplitude	0.06724	1	0.06724	6.86	**0.0396**
B: treatment time	1.64025	1	1.64025	167.30	**0.0000**
C: temperature	0.78961	1	0.78961	80.54	**0.0001**
AA	0.00505611	1	0.00505611	0.52	0.4997
AB	0.0003125	1	0.0003125	0.03	0.8642
AC	0.0001125	1	0.0001125	0.01	0.9182
BB	0.00627657	1	0.00627657	0.64	0.4541
BC	0.0120125	1	0.0120125	1.23	0.3107
CC	0.00627657	1	0.00627657	0.64	0.4541
Total error	0.0588246	6	0.00980409		
Total (corr.)	2.6167	15			

R-squared=97.752 %
R-squared (adjusted for df)=94.3799 %
Standard error of estimate=0.0990156
Mean absolute error=0.0452328
Durbin-Watson statistics=1.79552 (p=0.5797)
Lag 1 residual autocorrelation=0.0634847

Table 5. Analysis of variance (ANOVA) for ultrasound treatments and viability of *Escherichia coli*

Source	Sum of squares	df	Mean square	F-value	p-value
A: amplitude	0.09216	1	0.09216	34.40	**0.0011**
B: treatment time	1.92721	1	1.92721	719.34	**0.0000**
C: temperature	1.35424	1	1.35424	505.47	**0.0000**
AA	0.00668213	1	0.00668213	2.49	0.1654
AB	0.0000125	1	0.0000125	0.00	0.9478
AC	0.0066125	1	0.0066125	2.47	0.1672
BB	0.0414208	1	0.0414208	15.46	**0.0077**
BC	0.0045125	1	0.0045125	1.68	0.2420
CC	0.00429122	1	0.00429122	1.60	0.2526
Total error	0.0160749	6	0.00267915		
Total (corr.)	3.52224	15			

R-squared=99.5436 %
R-squared (adjusted for df)=98.859 %
Standard error of estimate=0.0517605
Mean absolute error=0.0257931
Durbin-Watson statistics=2.05114 (p=0.7815)
Lag 1 residual autocorrelation=–0.102588

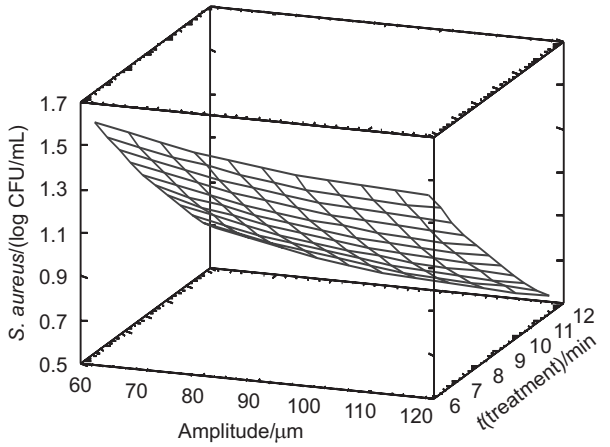

Fig. 1. Surface plot for *Staphylococus aureus* count at the optimum temperature (60 °C)

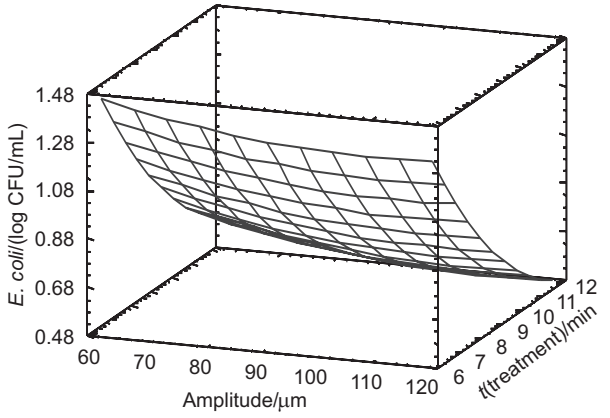

Fig. 2. Surface plot for *Escherichia coli* count at the optimum temperature (60 °C)

served that the count of *S. aureus* is the lowest after the treatment time of 12 min, temperature of 59.99 °C and at amplitude of 117.27 μm (Table 6). Similarly, Fig. 2 reveals that the count of *E. coli* was the lowest after the treatment time of 12 min, temperature of 59.99 °C and at amplitude of 110.41 μm (Table 7). At shorter treatment times and lower temperatures, the count of *S. aureus* and *E. coli* is higher. As discussed, this behaviour can be explained by the decreased cavitation effects and microstreaming with shorter treatment times.

Table 6. Optimised values of specific ultrasound parameters defined by STATGRAPHICS where the lowest count of *Staphylococcus aureus* was found

Factor	Low	High	Optim.	Optimum (the lowest) value of *S. aureus* count at this point
Amplitude/μm	60.0	120.0	117.27	
t(treatment)/min	6.0	12.0	12.0	0.5408
Temperature/°C	20.0	60.0	59.99	

Table 7. Optimised values of specific ultrasound parameters defined by STATGRAPHICS where the lowest count of *Escherichia coli* was found

Factor	Low	High	Optim.	Optimum (the lowest) value of *E. coli* count at this point
Amplitude/μm	60.0	120.0	110.41	
t(treatment)/min	6.0	12.0	12.0	0.4822
Temperature/°C	20.0	60.0	59.99	

Conclusions

The aim of the study was to investigate the effect of ultrasound and temperature on bacterial inactivation. Results of this investigation of the effect of combination of ultrasound and heat treatment *vs.* ultrasound treatment alone on the inactivation of *Escherichia coli* and *Staphylococcus aureus* in milk clearly indicate improved inactivation of both bacteria by the combined treatment. The parameters that seem to substantially affect the inactivation of *E. coli* and *S. aureus* in milk are the amplitude of the ultrasonic waves, the exposure/contact time with the microorganisms, and the temperature of the treatment. The results also indicate an increased inactivation of microorganisms under longer treatment times, higher temperatures and higher amplitudes applied. It was found that Gram-negative bacteria (*E. coli*) are more susceptible to the ultrasonic treatment than the Gram-positive ones (*S. aureus*). Output optimal values for achieving the maximum inactivation of *E. coli* and *S. aureus* were determined by STATGRAPHICS: temperature of 59.99 °C, treatment time of 12 min, and amplitude of 117.27 μm for *S. aureus* and of 110.41 μm for *E. coli*.

References

1. M.J.W. Povey, T.J. Mason: *Ultrasound in Food Processing*, Blackie Academic&Professional, London, UK (1998) pp. 53–57.
2. R.G. Earnshaw, J. Appleyard, R.M. Hurst, Understanding physical inactivation processes: Combined preservation opportunities using heat, ultrasound and pressure, *Int. J. Food Microbiol. 28* (1995) 197–219.
3. T.J. Mason, L. Paniwnyk, F. Chemat: Ultrasound as a Preservation Technology. In: *Food Preservation Techniques*, P. Zeuthen, L. Bøgh-Sørensen (Eds.), CRC Press, Boca Raton, FL, USA (2003) pp. 303–337.
4. P. Butz, B. Tauscher, Emerging technologies: Chemical aspects, *Food Res. Int. 35* (2002) 279–284.
5. M. Cameron, L.D. McMaster, T.J. Britz, Electron microscopic analysis of dairy microbes inactivated by ultrasound, *Ultrason. Sonochem. 15* (2008) 960–964.
6. P. Piyasena, E. Mohareb, R.C. McKellar, Inactivation of microbes using ultrasound: A review, *Int. J. Food Microbiol. 87* (2003) 207–216.
7. M. Villamiel, P. de Jong, Inactivation of *Pseudomonas flouorescens* and *Streptococcus thermophilus* in Trypticase® Soy Broth and total bacteria in milk by continuous-flow ultrasonic treatment and conventional heating, *J. Food Eng. 45* (2000) 171–179.

8. S. Patil, P. Bourke, B. Kelly, J.M. Frías, P.J. Cullen, The effects of acid adaptation on *Escherichia coli* inactivation using power ultrasound, *Innov. Food Sci. Emerg. Technol. 10* (2009) 486–490.

9. F.J. Sala, J. Burgos, S. Condón, P. Lopez, J. Raso: Effect of Heat and Ultrasound on Microorganisms and Enzymes. In: *New Methods of Food Preservation*, G.W. Gould (Ed.), Aspen Publishers, Gaithersburg, MD, USA (1995) pp. 176–204.

10. A. López-Malo, S. Guerrero, S.M. Alzamora, *Saccharomyces cerevisiae* thermal inactivation kinetics combined with ultrasound, *J. Food Prot. 62* (1999) 1215–1217.

11. R. Pagán, P. Mañas, J. Raso, S. Condón, Bacterial resistance to ultrasonic waves under pressure at nonlethal (manosonication) and lethal (manothermosonication) temperatures, *Appl. Environ. Microbiol. 65* (1999) 297–300.

12. J. Raso, R. Pagán, S. Condón, F.J. Sala, Influence of the temperature and pressure on the lethality of ultrasound, *Appl. Environ. Microbiol. 64* (1998) 465–471.

13. D.J. D'Amico, T.M. Silk, J. Wu, M. Guo, Inactivation of microorganisms in milk and apple cider treated with ultrasound, *J. Food Prot. 69* (2006) 556–563.

14. E. Ugarte-Romero, H. Feng, S.E. Martin, K.R. Cadwallader, S.J. Robinson, Inactivation of *Escherichia coli* with power ultrasound in apple cider, *J. Food Sci. 71* (2006) 102–108.

15. M.H. Dehghani, Effectiveness of ultrasound on the destruction of *E. coli*, *Am. J. Environ. Sci. 1* (2005) 187–189.

16. D. Knorr, M. Zenker, V. Heinz, D. Lee, Applications and potential of ultrasonics in food processing, *Trends Food Sci. Technol. 15* (2004) 261–266.

17. Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Enumeration of β–Glucuronidase--Positive *Escherichia coli*, Part 2, ISO 16649-2:2004, International Organization for Standardization, Geneva, Switzerland (2004).

18. Microbiology of Food and Animal Feeding Stuffs – Horizontal Method for the Enumeration of Coagulase-Positive Staphylococci, Part 1, ISO 6888-1:2004, International Organization for Standardization, Geneva, Switzerland (2004).

19. M.A. Margulis, I.M. Margulis, Calorimetric method for measurement of acoustic power absorbed in a volume of a liquid, *Ultrason. Sonochem. 10* (2003) 343–345.

20. Z. Herceg, E. Juraga, B. Sobota-Šalamon, A. Režek-Jambrak, Inactivation of mesophilic bacteria in milk by means of high intensity ultrasound using response surface methodology, *Czech J. Food Sci. 30* (2012) 108–117.

21. D.C. Montgomery: *Design and Analysis of Experiments*, John Wiley & Sons, New York, NY, USA (2001).

22. R.H. Myers, D.C. Montgomery: *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*, John Wiley & Sons, Hoboken, NJ, USA (2002).

23. R.O. Kuehl: *Design of Experiments: Statistical Principles of Research Design and Analysis*, Duxbury Press, Pacific Grove, CA, USA (2000) pp. 2–225.

24. A.I. Khuri, J.A. Cornell: *Response Surfaces: Design and Analyses*, Marcel Dekker, New York, NY, USA (1996).

25. C.H. Lu, N.J. Engelmann, M.A. Lila, J.W. Erdman Jr., Optimization of lycopene extraction from tomato cell suspension culture by response surface methodology, *J. Agric. Food Chem. 56* (2008) 7710–7714.

26. Regulations of Microbiological Standards for Foodstuffs, *Official Gazette of the Republic of Croatia, 20/2001*, Zagreb, Croatia (2001) (in Croatian).

27. S. Guerrero, A. López-Malo, S.M. Alzamora, Effect of ultrasound on the survival of *Saccharomyces cerevisiae:* Influence of temperature, pH and amplitude, *Innov. Food Sci. Emerg. Technol. 2* (2001) 31–39.

28. N. Gera, S. Doores, Kinetics and mechanism of bacterial inactivation by ultrasound waves and sonoprotective effect of milk components, *J. Food Sci. 76* (2011) 111–119.

29. M.L. Garcia, J. Burgos, B. Sanz, J.A. Ordoñez, Effect of heat and ultrasonic waves on the survival of two strains of *Bacillus subtilis*, *J. Appl. Bacteriol. 67* (1989) 619–628.

30. L. Ciccolini, P. Taillandier, A.M. Wilhem, H. Delmas, P. Strehaiano, Low frequency thermo-ultrasonication of *Saccharomyces cerevisiae* suspensions: Effect of temperature and of ultrasonic power, *Chem. Eng. J. 65* (1997) 145–149.

31. F. Noci, M. Walkling-Ribeiro, D.A. Cronin, D.J. Morgan, J.G. Lyng, Effect of thermosonication, pulsed electric field and their combination on inactivation of *Listeria innocua* in milk, *Int. Dairy J. 19* (2009) 30–35.

32. A. Patist, D. Bates, Ultrasonic innovations in the food industry: From the laboratory to commercial production, *Innov. Food Sci. Emerg. Technol. 9* (2008) 147–154.

33. G. Scherba, R.M. Weigel, W.D. O'Brien Jr., Quantitative assessment of the germicidal efficacy of ultrasonic energy, *Appl. Environ. Microbiol. 57* (1991) 2079–2084.